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14. ABSTRACT Prostate cancer (PCa) remained the largest diagnosed and second leading cause of cancer related deaths in men in the USA. Last year alone, may have recorded deaths of 28,170 American men by castration resistant prostate cancer (CRPC) (Siegel <i>et al.</i> 2012, 2013). Therefore developing new therapeutics that improves treatment options for this disease is urgently needed.					
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INTRODUCTION: W81XWH-11-1-0204

Prostate cancer (PCa) remained the largest diagnosed and second leading cause of cancer related deaths in men in the USA. Last year alone, may have recorded deaths of 28,170 American men by castration resistant prostate cancer (CRPC) (Siegel *et al.* 2012, 2013). Therefore developing new therapeutics that improves treatment options for this disease is urgently needed.

A large number of genetic, epigenetic and environmental factors contribute to the risk of prostate cancer. Among them are androgens, dietary factors, life-style related factors and genetic predisposition (Yegnasubramanian *et al.* 2004; Kim *et al.* 2012). A plethora of studies have contributed to our understanding with respect to molecular mechanisms, signaling pathways and intrinsic factors which contribute to the development of prostate cancer (PCa) and its subsequent transition to CRPC. A number of genes have been identified and characterized which are associated with inherited susceptibility of prostate cancer (De Marzo *et al.* 2003; Nelson *et al.* 2003; Nelson *et al.* 2009).

Male Androgen hormones (Testosterone, T; and Dihydrotestosterone, DHT) plays a pivotal role in the development, regulation, and maintenance of male phenotype as well as reproductive physiology and has been implicated in the development and progression of prostate cancer.

Androgen stimulates the expression of its cognate the Androgen nuclear receptor (Grossmann *et al.* 2001; Debes & Tindall 2002, 2004; Dehm & Tindall 2005, 2007). The Androgen receptor (AR) is a member of the family of intracellular steroid hormone receptors that function as transcription factor in a ligand-dependent (T and DHT) manner (Huang & Tindall 2002; Heemers *et al.* 2009; Lamont & Tindall 2010). Binding of DHT to AR facilitate the AR dimerization and the localization of the dimer protein to the nucleus. In the nucleus, AR binds to a large number of genes which contains Androgen Responsive Elements in the promoter region. The AR binding to a subset of AR-target gens triggers a transcriptional program that induces a myriad of cellular effects including proliferation, cell survival, differentiation, and secretion (Grossmann *et al.* 2001; Huang & Tindall 2002; Heemers & Tindall 2007). Emerging data suggests that constitutive sustained expression of AR may lead to activation of compensative cellular processes which facilitates the development of CRPC (Craft & Sawyers 1998; Chen *et al.* 2004; Chen *et al.* 2008). AR is prominent target for the treatment of non-organ confined prostate cancer by hormonal blockade therapy which uses anti-

androgens to competitively inhibits the binding of androgen to the ligand binding domain of the receptor (Craft & Sawyers 1998; Chen *et al.* 2008).

The AR is heterogeneously expressed in primary tumors and throughout the progression of hormone-sensitive and CRPC. In prostate carcinogenesis, changes in AR signaling pathways activate the growth of malignant cells (Chen *et al.* 2004). The hormone-refractory stage of the disease is commonly associated with the constitutive activation of AR expression by yet unknown molecular mechanisms (Craft & Sawyers 1998; Craft *et al.* 1999). In addition, it has also been suggested that AR may be targeting and modifying cellular factors to develop resistance and circumvent AR targeting therapies. A recent research suggests that AR can be targeted by andro-miRs in CRPC adjuvant therapeutics (Sikand *et al.* 2011a; Sikand *et al.* 2011b). It has also envisioned that resistance to treatment lead to alteration of AR role which modify the intrinsic microenvironment to modify behavior of certain cellular factors (Chen *et al.* 2004; Chen *et al.* 2008; Perets *et al.* 2012; Balbas *et al.* 2013).

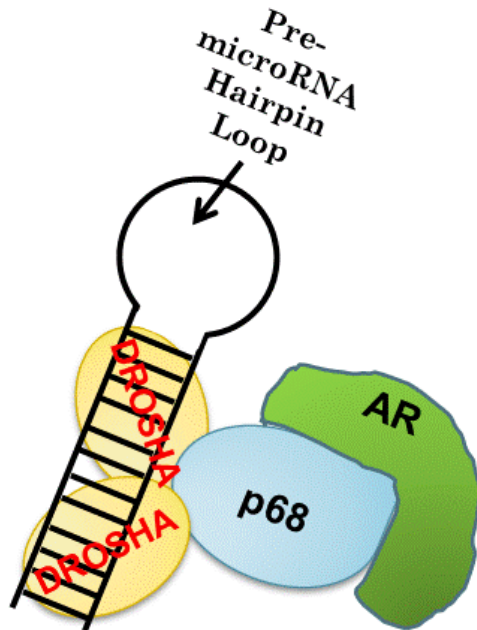
AR appears to interact with hundreds of cellular factors which include a large number of genomic loci and scores of protein factors implicated in Prostate carcinogenesis. The role of androgen and the AR appears to modulate the switching to autocrine pathway in favor of the paracrine loop. This pivotal switch appears to be played by AR interactions with cellular factors (Rajan *et al.* 2011; Perets *et al.* 2012; Vainio *et al.* 2012; Beltran *et al.* 2013; Imberg-Kazdan *et al.* 2013). One of the molecular mechanisms by which a prostate cancer cell survives and metastasizes after androgen-ablation therapy, perhaps involves the noncanonical functions of the AR including RNA processing and RNA-Protein interactions.

BODY: Based on some earlier observations that the AR was found in a complex associated with p68 helicase protein, which in turn interacts with the Drosha (Denli *et al.* 2004; Clark *et al.* 2008a; Clark *et al.* 2008b). Drosha, is a class III ribonuclease which function to process precursor-microRNAs (pre-miRNAs) to mature miRNA (Denli *et al.* 2004; Sohn *et al.* 2007; Mueller *et al.* 2010). miRNAs are class of small RNA which negatively regulates their target mRNAs by binding to the 3' UTR via hydrogen bonding. An earlier report showed that RNA helicase p68 is a coregulator of AR function in prostate cancer cells (Clark *et al.* 2008a; Clark *et al.* 2008b; Clark *et al.* 2013). Since, it is known that p68 RNA helicase interacts with the Drosha enzymes, we had proposed that AR might be modifying the post-transcriptional processing of “andro-miR” miRNAs

which have potential to contribute to the development of resistance to PCa therapies (Sikand *et al.* 2011a; Sikand *et al.* 2011b; Ebron 2013). miRNAs fine-tune gene expression at translational level by binding to its target mRNA 3' UTR and repressing protein synthesis. Cancer related miRNA studies are mainly focused on identifying miRNAs which target expression of genes implicated in various cancers including prostate cancer (PCa).

Proposal/Hypothesis: We had proposed potential protein:protein interactions among AR/p68 RNA Helicase/Drosha RNase III proteins in order to prevent the processing of “Andro-miRs,” the

SCHEMATIC OF THE PROPOSED HYPOTHESIS



The figure recapitulates the hypothesis i.e. pre-miRNA are bound to Drosha which also interacts with p68RNA helicase. P68 provides a bridge between AR and Drosha to facilitate processing of a subset of miRNAs

miRNA which are targeting AR expression.

One and only proposed specific aim and subaims of the study was **to identify miRNAs associated with AR/p68/Drosha complex.**

Stepwise, the proposed study was designed

1. to detect and confirm the existence of tripartite protein complex containing AR/p68 RNA Helicase/Drosha RNase III;
2. to optimize experimental conditions for the enrichment and isolation of the complex containing all three proteins;
3. to assay if the complex contains miRNAs;
4. and if the complex contains RNA component, we needed to establish the identify these interacting miRNAs either by miRNA microarray or by small RNA cloning and sequencing methods.

Aim: To identify miRNAs associated with AR/p68/Drosha complex: Following are sub-aims.

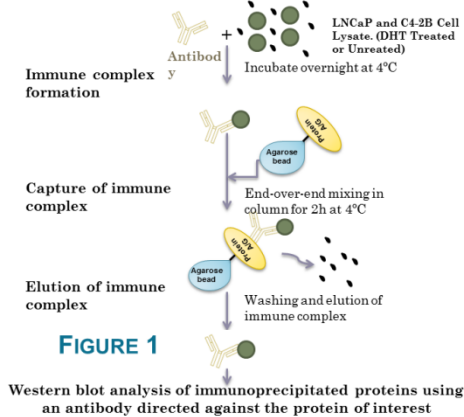
- 1a. Optimization of RIP-ChIP assay to pull-down AR/p68/Drosha complex to recover bound small RNAs.
- 1b. to clone small RNAs to generate cDNA libraries for sequencing.
- 1c. Sequencing and identification of miRNAs and miRNA microarrays to identify novel and existing known (albeit processed by AR/p68/Drosha complex) miRNAs, respectively, and
- 1d. to validate the intrinsic association properties of these miRNA with the AR/p68/Drosha complex by RNAi.

TASK 1: RNA Immunoprecipitation (RIP) of AR/p68/Drosha complex bound to primary or precursor miRNAs

- A. Optimization of RIP in LNCaP cells (Months 1-3).
- B. Optimization of RIP in C4-2B cells (Months 1-3).
- C. Isolation of AR/p68/Drosha complex enriched with pri- and/or pre-miRNAs and other noncoding RNAs (Months 3-6).

Task 1A: Optimization of RIP assay to pull-down AR/p68/Drosha complex to recover bound small RNAs.

CO-IMMUNOPRECIPITATION OF P68/ANDROGEN RECEPTOR/DROSHA COMPLEX



What we have done to accomplish the goals of the Task 1a: This task was the most crucial and the other subaims were basically dependent on the successful completion of the task1a. In this task we attempted to optimize RIP/ChIP in LNCaP and C4-2B cells for several months. During the earlier phase of the study a postdoctoral fellow attached to this project left the institute to pursue a faculty position around the starting time of the grant in 2011. Later, I

hired a graduate student in the January of 2012 consequently our projected time was delayed by several months. The student was able to optimize the Co-IP component of the RNA immunoprecipitation experiments. Here we present representative dataset from all the experiments performed to accomplish the task 1a.

Optimization and Experimental conditions for CoIP:

The CoIP experiments were done essentially following the previously published conditions (Berggard *et al.* 2007; Falsone *et al.* 2008; Jedamzik & Eckmann 2009). In brief, The cells were lysed using IP lysis buffer (Pierce Classic IP Kit - 26146). The immune complex was formed by incubating 10 µg of antibody (AR(441) sc-7305 mouse monoclonal IgG1/p68 RNA helicase(D-7) sc-365164 mouse monoclonal IgG2a , Santa Cruz Biotechnology/ Drosha (D28B1) rabbit mAb, Cell Signalling) with 500 µg of total protein lysate at 4 °C overnight. The immune complex formed with desired protein and antibody was captured using protein A/G agarose resin. The resin was washed and incubated with antibody/lysate complex in the spin column. The mixture was incubated at 4 °C with end-over-end shaking for 2h. The resin was washed to remove nonspecific proteins and the immune complex was eluted using Non reducing sample buffer. The protein complex is boiled at 100 °C and then applied on SDS-PAGE gel for further analysis.

Based on published evidences we had predicted the presumptive existence of AR/p68/Drosha complex and its putative role in processing of small RNAs (Clark *et al.* 2008a; Clark *et al.* 2008b). To test this hypothesis, first we needed to optimize and perform Co-IP using whole cell lysates from LNCaP and C4-2B cells. We pulled down protein:protein interacting complexes using one of three antibodies (AR or p68 RNA Helicase or Drosha) and probed the presence of other two proteins in Western blots. This was necessary to establish the biochemical existence of the tripartite complex is in PCa cells. We used LNCaP and C4-2B cell lines because they represent the preclinical models of AR-positive androgen-dependent and AR-positive androgen-independent stage of prostate cancer (Thalmann *et al.* 1994; Thalmann *et al.* 2000; Denmeade *et al.* 2003a; Denmeade *et al.* 2003b; Liu *et al.* 2004).

Results: Results for Co-IP using AR or p68 Helicase or Drosha are essentially the same results that we submitted last year. These results show our efforts directed towards the optimization of the protocols. The main idea of these experiments was to pull down complex containing AR/p68 RNA Helicase/Drosha with AR antibodies. To test our hypothesis we performed numerous Co-IPs using antibodies against the three proteins. If the case of the presence of tripartite proteins' complex we expected to see the detection of all three proteins using a single type antibody directed towards the one member of the complex. So in theory single antibody has potential to pulldown the tripartite

protein complex containing all three proteins.

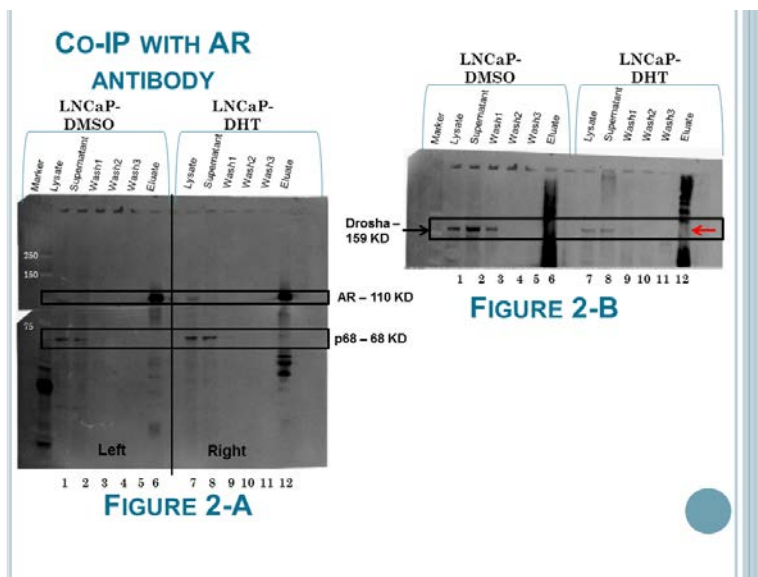


Figure 2: Results of Co-IP performed by using anti- AR antibody: Western blots were probed with anti-p68 helicase or anti-Drosha antibodies. Figure 2A-Left and right panel show the results of Co-IP done using lysates prepared from LNCaP cells treated with vehicle (DMSO) or DHT, respectively. DHT treatment was done to enrich the putative tripartite protein complex. In Right panel of Fig 2-A, Lane 12 show that p68 RNA Helicase was precipitated with AR antibody. In addition, we also observed very faint band of Drosha (expected molecular weight 159 kd) (Figure 2-B lane 12 marked

by Red Arrow).

Detection of p68 RNA helicase and Drosha protein in AR antibody pull down experiment:

First we optimized protein complex pull-downs using AR antibodies and probing the western blot using either AR (as a positive control) Drosha or p68 RNA helicase antibodies in LNCaP cell line. Cells were treated with appropriate concentrations of DHT to ensure the activation and nuclear localization of AR and enrichment of AR/p68/Drosha nuclear complexes. As results show in Figure 2, AR Co-IP pull down experiment was able to detect p68 (Fig 2, Lower Right Panel A, Lane 12 in DHT enriched LNCaP cells). In addition, we found a faint band when the blot was probed with Drosha antibody (Figure 2B, Lane 12, Red arrow). It is interesting to observe that DHT treatment was able to enrich the complex for Drosha. However, the enrichment wasn't sufficient enough to detect higher concentration of Drosha. Since the experimental conditions were mild the complex contains many other proteins that are cross reacting with Drosha antibody.

Detection of Drosha and AR protein in p68 RNA helicase antibody pull down experiment:

Next we optimized protein complex pull-downs from LNCaP cell lysates using p68 antibodies and probed the complex for the presence of AR and Drosha proteins. LNCaP and C4-2B Cells were appropriately treated with DHT for the activation of AR and enrichment of putative AR/p68/Drosha nuclear complexes. Results of the experiments are shown in the Figure 3. Figure legend describes

the details of the results.

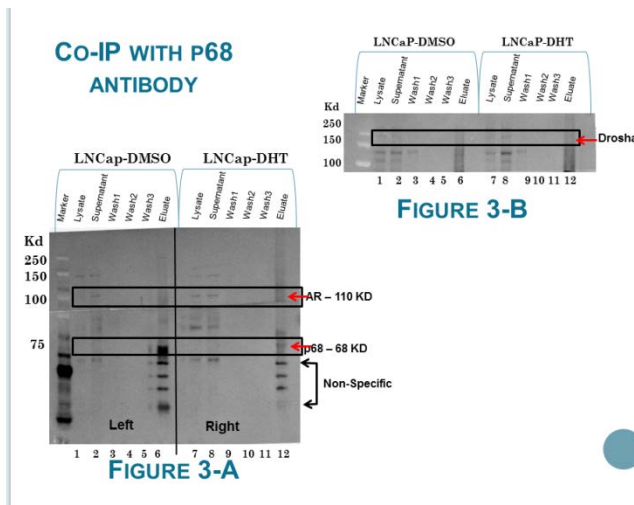


Figure 3: Detection of AR and Drosha proteins in p68 pull-down experiment: The figure shows results of Co-IP performed using anti-p68 RNA Helicase antibody. The blots were probed with anti-AR (Figure 3A-Right top LNCaP-DHT Panel, Lane 12) or with anti-Drosha (Figure 3B-LNCaP-DHT Panel, Lane 12, marked by a red arrow).

The data indicates the presence of AR and Drosha proteins in the complex as shown in Figure 3. Figure 3A-Right top LNCaP-DHT Panel, Lane 12. In addition, we also detected a

very signal for Drosha in Figure 3B-LNCaP-DHT Panel, Lane 12.

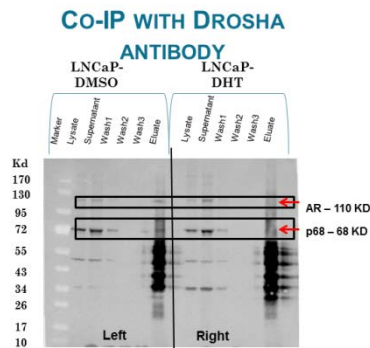


FIGURE 4

Finally, we attempted to pull-down putative AR/p68 RNA Helicase/Drosha complex using Drosha antibody and probed the blots for the presence of with AR and p68 RNA helicase.

Figure 4: The figure show results of Co-IP performed using anti-Drosha antibody. The western blots were probed with anti-AR and anti-p68 antibodies. Please see the presence of p68 band in lower red-box in LNCaP-DHT panel Elute Lane.

Summary of the Results discussed above: The above experiments suggested the presence of AR/p68 RNA helicase/Drosha RNase III enzymes in the tripartite protein:protein complex. The results show that proteins (all three) are detectable most in DHT enriched lysates. This result suggests that the protein complex is highly enriched in DHT mediated translocation of AR in nucleus. We are able to detect the presence of all three proteins in three co-IP experiments. However, the presence of Drosha in AR or presence of AR in Drosha pulldown experiments needs to be confirmed by an alternative experiment. This experiment can be performed using ectopically expressing recombinant proteins in any cell line.

TASK 2: Identification of RIP miRNAs and other noncoding RNAs: The success of this task was mainly dependent on the success of the task 1. We needed to establish conditions for highly enrichment of the tripartite protein complex. However, our repeated attempts to enrich RNA:Protein complexes from the lysates prepared from the cells were futile. Due to the lack of any Drosha protein in the proposed tri-protein complex our attempts to continue to purify RNA did not work as anticipated.

Subtasks proposed but not accomplished:

- A. Sequencing of library and identification of RNAs by bioinformatics;
- B. primary miRNA microarray; and

- C. Validation of intrinsic association of miRNA with the novel AR/p68/Drosha complex will be established by RNAi of AR and/or p68 genes.

RIP-ChIP -

RIP-ChIP was essentially performed using the conditions that are described in original protocols (Keene *et al.* 2006; Mukherjee *et al.* 2011; Erhard *et al.* 2013). To perform RIP in LNCaP or C4-2B cells to isolate small RNA, we developed a protocol to pull down and clone RNA bound to AR/p68/Drosha complex for sequencing. We cloned fraction of RNAs isolated from protein:RNA complex in TOPO vectors. However, sequencing of a large number of clones did not reveal any novel or known RNAs that we can pursue further.

Key Research Accomplishments: During the span of this grant we tested our hypothesis if AR binds to p68 and Drosha to modify processing of certain miRNAs. In this project we were able to identify interaction between the AR and p68 RNA helicase. However, we were unable to find any indication that Drosha bound to p68 also have AR bound to this complex. We made serious attempts to optimize the CO-IP protocols, however our repeated efforts did not yield any data that show triple protein complex bound to RNA. As such there are not tangible key research accomplishments that can be able to report at this time.

Reportable Outcomes: We have data that indicates the intrinsic association of AR with p68 RNA helicase but not with Drosha. However, there is not enough data that could be used for any extended studies for the moment.

Conclusion: During the previous reporting session, we were able to optimize CO-IP methods to pull-down AR/p68/Drosha protein:protein interacting complex in LNCaP cells. For the past one year, we extended the study on C4-2B cells however unable to identify any novel interacting complex that we had proposed in the original grant application. We did try to isolate and clone, if any small RNAs from pull down complexes, but without any luck. We hereby conclude, that the

current data does not support our hypothesis that AR has potential to modify expression of a subset of miRNAs involved in prostate carcinogenesis.

APPENDICES: No Appendix is currently needed for this report.

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